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(71) Applicant *(for all designated States except US)*: THE WELLCOME FOUNDATION LIMITED [GB/GB]; Unicorn House, 160 Euston Road, London NW1 2BP (GB).

(72) Inventor; and
(75) Inventor/Applicant *(for US only)* : CHARLES, Ian, George [GB/GB]; The Wellcome Foundation Limited, Langley Court, Beckenham, Kent BR3 3BS (GB).

(74) Agents: WOODS, Geoffrey, Corlett et al.; J.A. Kemp & Co., 14 South Square, Gray's Inn, London WC1R 5LX (GB).

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(54) Title: ACELLULAR VACCINE

(57) Abstract

A proteine which is uncontaminated by components from *B. parapertussis*, which is capable of binding to antibody which also binds the native P70 antigen of *B. parapertussis* and which has (a) the amino acid sequence shown in Figure 1, from amino acid residue Asp 35 to Asn 643 or (b) an amino acid sequence which has homology of more than 98 % with the said amino acid sequence (a), DNA encoding the protein, expression vectors containing said DNA, host cells transformed by such expression vectors and the use of the antigen in a vaccine for the prevention of *B. parapertussis* infections.

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ACELLULAR VACCINE

The invention relates to the isolation and characterisation of an antigen of Bordetella parapertussis and the antigenic sites, DNA sequences encoding the antigen, expression vectors containing such DNA sequences, host cells transformed by such expression vectors. The invention also relates to the use of the antigen in a vaccine for the prevention of B.parapertussis infections.

B.pertussis causes a serious and debilitating disease in humans, especially children. It has been kept under control in the developed countries by large scale immunisation programmes. Traditionally, immunisation has been carried out using a whole cell vaccine derived from cell cultures of B.pertussis and has been found to be relatively effective in preventing the disease.

Concern over adverse reactions, such as fever, local reactions and persistent screaming, has led in recent years to a reduced acceptance of the whole cell B.pertussis vaccine and debate about its continued use. Research has therefore been directed towards the development of vaccines which lack the components responsible for the adverse effects of the whole cell vaccines, whilst retaining the components necessary to confer protection against the disease.

The search for safe and effective vaccines has been hampered by the paucity of information regarding the identity and mechanisms of action of the pathogenic, toxic and protective moieties of the bacterial organism contained in whole cell vaccines. The search for effective vaccines has, therefore, focused on the isolation and characterisation of surface antigens of the organism and on their efficacy in inducing an immune reaction (J.Am.Med.Soc., 1982, 248(1) 22-23). Examples of B.pertussis antigens that have been investigated include lymphocytosis promoting factor (LPF, otherwise known as pertussis toxin (PT)), filamentous haemagglutinin (FHA), lipopolysaccharide (LPS), agglutinogens, dermonecrotic toxin (DNT), heat labile and heat stable

toxins, polymorphonuclear leukocyte inhibitor factor, adenylate cyclas , the outer membrane 69kDa antigen (P.69, pertactin) and other surface components.

B.parapertussis is closely related to B.pertussis and is also responsible for outbreaks of whooping cough in man (Zeulzer et al, J. Pediatr. 9:493-497 (1946); Linneman et al J. Pediatr. 19: 229-240 (1977); Novotny, J.Infect. Dis. 161:581-582, 1990)). However, to date no vaccine specific for B.parapertussis has been developed.

B.parapertussis is also known to produce a number of virulence factors (Pitmann, Bergey's Manual of Systematic Bacteriology Vol I P388-393 Ed. Kreig & Hoft 1984) including FHA, and adenylate cyclase (AdeCase) toxin. Because of the immunological cross-reactivity of these virulence factors, vaccination against whooping cough with a whole cell vaccine may provide some protection against B.parapertussis. However, B.parapertussis does not produce the well characterised pertussis toxin (PT). Thus, outbreaks of B.parapertussis mediated whooping cough are unlikely to be prevented by acellular vaccines composed primarily of detoxified pertussis toxin (PT).

Furthermore, if vaccination programmes against B.pertussis are successful in reducing the incidence of the bacterial organism in the human population, or even in eradicating the organism altogether, just as smallpox has been eradicated by rigorous vaccination programmes, there is a danger that B.parapertussis infections will increase in number and B.parapertussis will become the primary cause of whooping cough. It is therefore desirable to include in any whooping cough vaccination programme, a B.parapertussis specific vaccine, preferably an acellular vaccine.

I.G. Charles et al Proc. Natl. Acad Sci USA Vol 80 pp 3554-3558 (1989) purport to identify homologous proteins in B.pertussis and B.parapertussis with molecular masses of 69 and 70kDa respectively. The assertion of homol gy is based on the observation that these

antigens bind to the BB05 antibody raised against B.bronchiseptica. These proteins although clearly related to each ther in their ability to bind BB05 antibody have different immunogenic properties. It is not stated whether the antigen of B.parapertussis would be expected to have any immunogenic potential in vivo, this being necessary for use as an effective vaccine.

The inventors have identified, isolated and characterised an antigen of B.parapertussis described hereinafter, which is useful as the basis for an accelullar vaccine specific for B.parapertussis or as part of an acellular whooping cough vaccine.

Accordingly, the invention provides a B.parapertussis antigen comprising an amino acid sequence as set out in Figure 1, or an amino acid sequence that is at least 94% homologous with that set out in Figure 1, or an antigenic fragment thereof.

The invention also provides a protein which is uncontaminated by components from B.parapertussis, which is capable of binding to antibody which also binds the native P70 antigen of B.parapertussis and which has the amino acid sequence as set out in Figure 1 from amino acid residue Asp 35-Asn 643, or an amino acid sequence that is at least 94% homologous with that set out in Figure 1 from amino acid residue Asp 35 - Asn 643, or an antigenic fragment thereof.

The invention also includes the amino acid sequence set out in Figure 1, or one which is at least 95% or even 98% homologous with that set out in Figure 1.

The amino acid sequence of Figure 1 shows a precursor protein of molecular weight approximately 95,000 Daltons (P.95) comprising 922 amino acids. In vivo processing of this precursor yields a protein with an estimated molecular weight of 70,000 Daltons (P.70) as determined by SDS polyacrylamide gel electrophoresis, but the actual weight is appr ximately 61 kDa.

The invention therefore provides as a preferred feature, the amino acid sequence of Figure 1 comprising the amino acid sequence from Asp-35 to Asn 643 or an antigenic fragment thereof.

An antigenic fragment of the amino acid sequence set forth in Figure 1 or of a sequence which is at least 94% homologous therewith preferably includes amino acids Pro577 to Pro 612 or Ala 574 to Pro 612.

The antigenic sites of the amino acid sequence set out in Figure 1 may be identified using standard procedures. These may involve fragmentation of the polypeptide itself using proteolytic enzymes or chemical agents and then determining the ability of each fragment to bind to antibodies or to provide an immune response when innoculated into an animal or suitable in vitro model system (Strohmauer et al, J.Gen.Virol, 1982, 59, 205-306). Alternatively, the DNA encoding the polypeptide may be fragmented by restriction enzyme digestion or other well-known techniques and then introduced into an expression system to produce fragments (optionally fused to a polypeptide usually of bacterial origin). The resulting fragments are assessed as described previously (Spence et al, J.Gen.Virol., 1989, 70, 2843-51; Smith et al, Gene, 1984, 29, 263-9). Another approach is to chemically synthesise short peptide fragments (3-20 amino acids long; conventionally 6 amino acids long) which cover the entire sequence of the full-length polypeptide with each peptide overlapping the adjacent peptide. (This overlap can be from 1-10 amino acids but ideally is n-1 amino acids where n is the length of the peptide; Geysen et al, Proc. Natl. Acad. Sci., 1984, 81, 3998-4002). Each peptide is then assessed as described previously except that the peptide is usually first coupled to some carrier molecule to facilitate the induction of an immune response. Finally, there are predictive methods which involve analysis of the sequence for particular features, e.g. hydrophilicity, thought to be associated with immunologically important sites (Hopp and Woods, Proc. Natl. Acad. Sci., 1981, 78, 3824-8; Berzofsky, Science, 1985, 229, 932-40). These predictions may

then be tested using the recombinant polypeptide or peptide approaches described previously.

The antigen of the present invention is located on the surface of B.parapertussis, and may be isolated from cultures of B.parapertussis by conventional methods, for example as described by Novotny et al (1985) Infection and Immunity 50, 199-206 and European Publication No. 0162639. Alternatively, it may be obtained using recombinant DNA technology. In this regard, the DNA encoding the antigen may be cloned, for example as described by Makoff et al (Bio-Technology November 1990), inserted into an expression vector which is used to enable expression in an appropriate host.

The invention therefore provides a cloned DNA sequence encoding the antigen of the present invention, preferably the DNA sequence is as set out in Figure 1 or is at least 92% homologous with that set out in Figure 1. The invention also includes a DNA sequence encoding the precursor protein namely from nucleotide 145 to nucleotide 2910 or a DNA sequence encoding the P70 protein namely from nucleotide 247 to nucleotide 2073.

The antigen is preferably provided in a pure form ie. greater than 90% most preferably greater than 95% pure but at least to a level consistent with its use in a human vaccine.

The cloning of the DNA sequence may be carried out using standard procedures known in the art. However, it is particularly advantageous in such procedures to employ the sequence data disclosed herein so as to facilitate the identification and isolation of the desired cloned DNA sequences. Preferably, the DNA is isolated by the method described by Hull et al Infect. Immun. 33: 933-938 (1981) as modified by Maskell et al J.Bacteriol. 170: 2467-2471 (1988). The DNA is then digested with a restriction enzyme to generate short fragments which are then inserted into a cloning vector, such as the cosmid pHC79 or a

derivative thereof and the resulting recombinant DNA molecules used to transf rm E.coli and thus generate the desired library.

The library may be screened using a standard screening strategy. For example one may employ as hybridisation probes one or more labelled oligonucleotides synthesised using the DNA sequence information disclosed herein. One or more additional rounds of screening of one kind or another may be carried out to characterise and identify positive clones.

Having identified a first positive clone, the library may be rescreened for additional positive clones using the first clone as an hybridization probe. Alternatively or additionally, further libraries may be prepared and these may be screened using hybridisation probes. In this way, further DNA sequences may be obtained.

Alternatively, the DNA sequence encoding the antigen of the present invention may be synthesised using standard procedures and this may be preferred to cloning the DNA in some circumstances.

Thus cloned or synthesised, the desired DNA sequence may be inserted into an expression vector using known and standard techniques. The expression vector is normally cut using restriction enzymes and the DNA sequence inserted using blunt-end or staggered-end ligation. The cut is usually made at a restriction site in a convenient position in the expression vector such that, once inserted, the DNA sequence is under the control of the elements of DNA that effect its expression.

These elements may vary according to the host but usually include a promoter, ribosome binding site, translational start and stop sites, and a transcriptional termination site. Examples of such vectors include plasmids and cosmids. Expression vectors of the present invention encompass both extrachromosomal vectors and vectors that are integrated into the host cell's chromosome.

The invention therefore provides an expression vector containing a DNA sequence, as herein defined, and being capable in an appropriate host of expressing the DNA sequence to produce the antigen.

The invention also provides a host cell transformed with an expression vector as herein defined.

Examples of host cells of use with the invention include prokaryotic and eukaryotic cells, such as bacterial, yeast, mammalian and insect cells. Particular examples of such cells are E.coli, S.cerevisiae, P.pastoris, Chinese hamster ovary and mouse cells, and Spodoptera frugiperda and Tricoplusia ni. The choice of host cell may depend on a number of factors but, if post-translational modification of the antigen is important, then a prokaryotic host would be preferred.

Transformation of a host cell may be carried out using standard techniques. Some phenotypic marker is usually employed to distinguish between the transformants that have successfully taken up the expression vector and those that have not. Culturing of the transformed host cell and isolation of the antigen may also be carried out using standard techniques.

The invention thus provides a process for preparing the antigen which comprises cloning or synthesising a DNA sequence encoding the antigen, as herein defined, inserting the DNA sequence into an expression vector such that it is capable in an appropriate host of being expressed, transforming a host cell with the expression vector, culturing the transformed host cell, and isolating the antigen.

The antigen obtained in this way may be insoluble and thus may need to be refolded following the use of guanidinium hydrochloride as denaturant in conventional manner and in any event is preferably purified.

The invention therefore provides a vaccine containing an antigen of B.parapertussis as hereinafter described.

The vaccine of the invention may optionally contain additional antigens of B.parapertussis or other bacteria, such as B.pertussis, tetanus and diphtheria.

The vaccine of the invention is normally associated with a pharmaceutically acceptable vehicle which allows the antigen to be administered to the patient. Administration is usually carried out via the oral, intranasal, or preferably parenteral route. In the case of the parenteral route, the vehicle is generally liquid and the antigen is generally dissolved or suspended in it. An example of a liquid vehicle is physiological saline solution.

The vaccine may also contain an adjuvant for stimulating the immune response and thereby enhancing the potency of the antigen. Convenient adjuvants for use in the present invention include, for example, aluminium hydroxide and aluminium phosphate.

Conveniently the vaccine contains a final concentration of antigen in the range of from 0.01 to 5mg/ml, preferably 0.03 to 2mg/ml, most preferably 0.3mg/ml. After formulation the vaccine may be incorporated into a sterile container which is then sealed and stored at a low temperature, for example 4°C, or is freeze-dried.

The invention also provides a method for inducing immunity to whooping cough in humans, comprising the administration to the patient of an effective amount of the vaccine of the present invention.

In order to induce immunity to whooping cough in humans one or more doses of the vaccine are normally administered. Each dose of the vaccine is 0.1 to 2ml preferably 0.2 to 1ml, most preferably 0.5ml.

The invention will now be exemplified further by reference to the accompanying Figures and the Examples.

Figure Legends

Figure 1. The amino acid sequence of P.70 and the DNA sequence of the prn gene encoding the P.70 antigen from B.parapertussis.

Figure 2. Restriction map and sequencing strategy for the prn gene encoding the P.70 antigen from B.parapertussis. Small arrows represent the direction and extent of DNA sequencing derived from restriction sites or oligonucleotides used as specific primers. The large arrow indicates the direction of transcription of the orf for the gene.

Figure 3. Line drawing showing the strategy used to generate the P.70 antigen expression plasmid pBD845.

Experimental procedures

Bacterial strains, plasmids and phage

B.pertussis strain CN2992, B.parapertussis strain CN2591 were from the Wellcome Culture Collection. E.coli K.12 TG1 [Δ (lac-pro) supE, thi, hsdD5/F' traD36 $proA^{+}B^{+}$, $lacI^{q}$ lacZ M15 was as described (Carter et al, (1985)] as was E.coli K12 HB101 F^{-}, hsdS20 ($r^{-}B$ $m^{-}B$) recA13, ara-14, proA2, lacY1, galK2, rpsL20, xyl-5, mtl-1, supE44 (Boyer & Roulland-Dussoix, J.Mol.Biol. 41:459-465, 1969).

Cosmid pHC79 (Hohn & Collins, Gene 11:29-298, 1980) was from Amersham International, Little Chalfont, Buckinghamshire; plasmid pKK223-2 (Amman & Brosius, Gene 40:183-90, 1985) and M13mp18 and M13mp19 (Yanisch-Perron et al, Gene 33:103-119, 1985) were supplied by Pharmacia Ltd., Milton Keynes, Buckinghamshire.

Media and reagents

E.coli strains were grown on Luria broth (LB) or on LB solidified with 1.6% (w/v) agar (Miller, Experiments in Mol.Gene. Cold Spring Harbor N.Y., 1972) (Difco Address). Minimal medium (MM) was made as described by Miller (1972) and was solidified with 2% (w/v) Noble agar (Difco). B.pertussis and B.parapertussis were grown in Stainer-Scholte broth at 37°C (Stainer & Scholte, J.Gen.Microbiol 63:211-220 1962), or on Stainer-Scholte plates solidified with 2% (w/v) Noble agar. Deoxynucleotides, ampicillin, tetracyline and dithiothreitol were from Sigma. Restriction endonucleases and T4 DNA ligase were from BRL, Paisley, Scotland.

Cloning of B.parapertussis chromosomal DNA into cosmid pHC79 and identification of the gene encoding P.70

B.parapertussis chromosomal DNA (prepared by the method of Hull et al Infect.Immun. 33:933-938, 1981 as modified by Maskell et al J.Bacteriol. 170:2467-2471, 1988) was partially digested with Sau3A, and fragments in the 40-50kb size range were ligated into the BamHI site (Maniatis et al Molecular Cloning: Cold Spring Harbor N.Y, 1982; of cosmid pHC79 (Hohn & Collins Gene 11:291-298, 1980). Recombinant cosmids in E.Coli HB101 were plated out and transferred to microtiter plates following the method described by Charles et al (J.Gen.Microbiol. 136:353-358, 1990) and transferred to Gene Screen Plus hybridized membranes (Du Pont, Stevenage, Hertfordshire). The cosmids were then screened for the presence of the prn gene encoding P.70 by DNA : DNA hybridization using a radioactively labelled 1.8kb ClaI restriction fragment isolated from the related prn gene from B.pertussis. The ClaI fragment was gel purified (Tautz & Renz, Anal. Biochem. 132:14-19, 1983) following digestion of cosmid pI69 (Charles et al Proc. Natl. Acad-Sci. USA 86:3554-3558, 1989). The fragment was nick translated with a kit supplied by BCL, Mannheim, and[α^{32}P]-ATP (Amersham), and hybridized with the B.parapertussis gene bank filters as previ usly described (Charles et al, J.Gen.Microbiol. 136: 353-358

(1990)). Three positive colonies were identified and one, harbouring cosmid pBD811 was selected for further analysis.

Subcloning and DNA sequencing

Restriction fragments of cosmid pBD811 were cloned in M13mp18 and M13mp19 (Yanisch-Perron et al, Gene 33:103-119, 1985) and sequenced using universal primer, [α^{-35}S] dATP (deoxyadenosine 5'-[α^{35}S] thiotriphosphate) dideoxynucleotide triphosphates, and both gradient and wedge gels (Biggin et al, Proc.Natl. Acad.Sci USA 80:3963-3965, 1983; Sanger et al, Proc.Natl. Acad.Sci. USA 71:5463-5467, 1977). To resolve compression artifacts some clones were sequenced with modified T7 DNA polymerase (Tabor & Richardson, Proc.Natl. Acad.Sci USA 84:4767-4771, 1987) and 7-deaza-2'-dGTP (Mizusawa et al, Nucl.Acids.Res. 14:1319-1324, 1986) using a kit supplied by Pharmacia. Gaps in the sequence were filled in using synthetic oligonucleotides as specific primers (Charles et al, Nucl.Acids.Res. 14:2201-2213, 1986).

Oligonucleotides for use as specific sequencing primers were made on a SAM1 oligonucleotide synthesizer (Biolabs).

Computer analysis of the DNA sequence revealed an open reading frame capable of encoding a protein of 922 amino acids with a calculated molecular weight of 95,177.

Expression of P.70 antigen from B.parapertussis on the Surface of E.coli

Using Western blotting, it was not possible to detect the expression of P.70 in E.coli HB101 harbouring cosmid pBD811, although from DNA sequence analysis the cosmid was known to contain the entire structural gene. In order to express the B.parapertussis prn gene in E.coli, the expression vector pKK233-2 (Amann & Brosius, Gene

40:183-90, 1985) was used, which directs high level transcription from a trc promoter.

A three-way ligation using NcoI-HindIII digested pKK233-2; a 1.6kb AflIII-EcoRV fragment from the 5' end of P.70 prn and a 2.2 kb EcoRV-HindIII fragment containing the 3' end of P.70 prn was carried out (see Fig. 3). The ligation mix was used to transform E.coli TG1 and transformants expressing P.70 antigen identified by their ability to cross-react with the mAb BB05 (Novotny et al, Develop.Biol.Stand. 61:27-41, 1985) in protein dot blots. Colonies returning a positive signal were then isolated, and miniplasmid preps (Maniatis et al, Molecular Cloning: A labaratory Manual, Cold Spring Harbor, N.Y., 1982) carried out to verify that a full-length insert had been cloned. One of the colonies, harbouring plasmid pBD845, was selected for further study. SDS-PAGE electrophoresis was followed by Western blotting. Samples were transferred to nitrocellulose by the method of Towbin (Proc.Natl.Acad-Sci USA 76:4350-4354, 1984). The detection system used was horse radish peroxidase conjugated goat anti-mouse IgG using 4-chloro-1-napthol as substrate (Fairweather et al, J.Bacteriol. 165:21-27, 1986). Western blotting of E.coli TG1 harbouring pBD845, (containing the entire structural gene for P.95), shows that a protein with a molecular weight of 95kDa (P.95) is weakly expressed in E.coli, along with a more significant band of 70kDa (P.70). A large number of lower molecular weight species that cross-react with BB05 are also seen suggesting that P.70/P.95 is unstable in E.coli. These observations suggest that the P.95 form of the protein is initially expressed and is subsequently processed to the P.70 form.

Slide agglutination assays. Samples of E.coli strains harbouring pBD845 to be tested (10^6-10^7) were mixed with 30μl of IgG purified polyclonal rabbit anti-P.69 antibody on a glass microscope slide and visually scored for clumping after 2 mins as compared with a negative control of either vir$^-$ B.parapertussis or E.coli TG1. E.coli strains harbouring pBD845 can be agglutined, and this agglutination occurs just as rapidly as with vir$^+$ B.parapertussis suggesting that

recombinant P.70 protein is correctly expressed on the surface of E.coli.

CLAIMS

1. A protein which is uncontaminated by components from B.parapertussis, which is capable of binding to antibody which also binds the native P.70 antigen of B.parapertussis and which has (a) the amino acid sequence shown in Figure 1 from amino acid residue Asp 35 to Asn 643, or (b) amino acid sequence which has a homology of more than 98% with the said amino acid sequence (a).

2. A DNA sequence which encodes a protein as defined in claim 1.

3. A DNA sequence according to claim 2, which consists essentially of the nucleotide sequence shown in Figure 1 from nucleotide 247 to nucleotide 2073.

4. A DNA sequence according to claim 2, which differs from the nucleotide sequence shown in Figure 1 from nucleotide 247 to nucleotide 2073 at no more than twelve positions.

5. A protein which has the amino acid sequence shown in Figure 1 or an amino acid sequence which has a homology of more than 98% with the amino acid sequence shown in Figure 1.

6. A DNA sequence which encodes a protein as defined in claim 5.

7. A DNA sequence according to claim 6, which consists essentially of the nucleotide sequence shown in Figure 1 from nucleotide 145 to nucleotide 2910.

8. A DNA sequence according to claim 6, which differs from the nucleotide sequence shown in Figure 1 from nucleotide 145 to 2910 at no more than twelve positions.

9. A DNA sequence which consists essentially of the sequence shown in Figure 1 or a s quence which has a homology of more than 98% with the sequence shown in Figure 1.

10. An expression vector which contains a DNA sequence as defined in any one of claims 2 to 4 and 6 to 9, and which, when provided in a suitable host, is capable of expressing a protein as defined in claim 1 to 5.

11. A vector according to claim 10, which is a plasmid.

12. A host transformed with an expression vector as defined in claim 10 or 11.

13. A host according to claim 12, which is a strain E.coli.

14. A process for the preparation of a protein as defined in claim 1, which process comprises maintaining a host as claimed in claim 12 or 13 under such conditions that said protein is expressed.

15. A process for the preparation of a protein as defined in claim 5, which process comprises maintaining a host as claimed in claim 12 or 13, which has been transformed by an expression vector as claimed in claim 10 which contains a DNA sequence as defined in any one of claims 6 to 9, under such conditions that the said protein is expressed.

16. A pharmaceutical composition comprising a pharmaceutically acceptable carrier or diluent and, as active ingredient, a protein as defined in claim 1 or 5.

Fig.1.

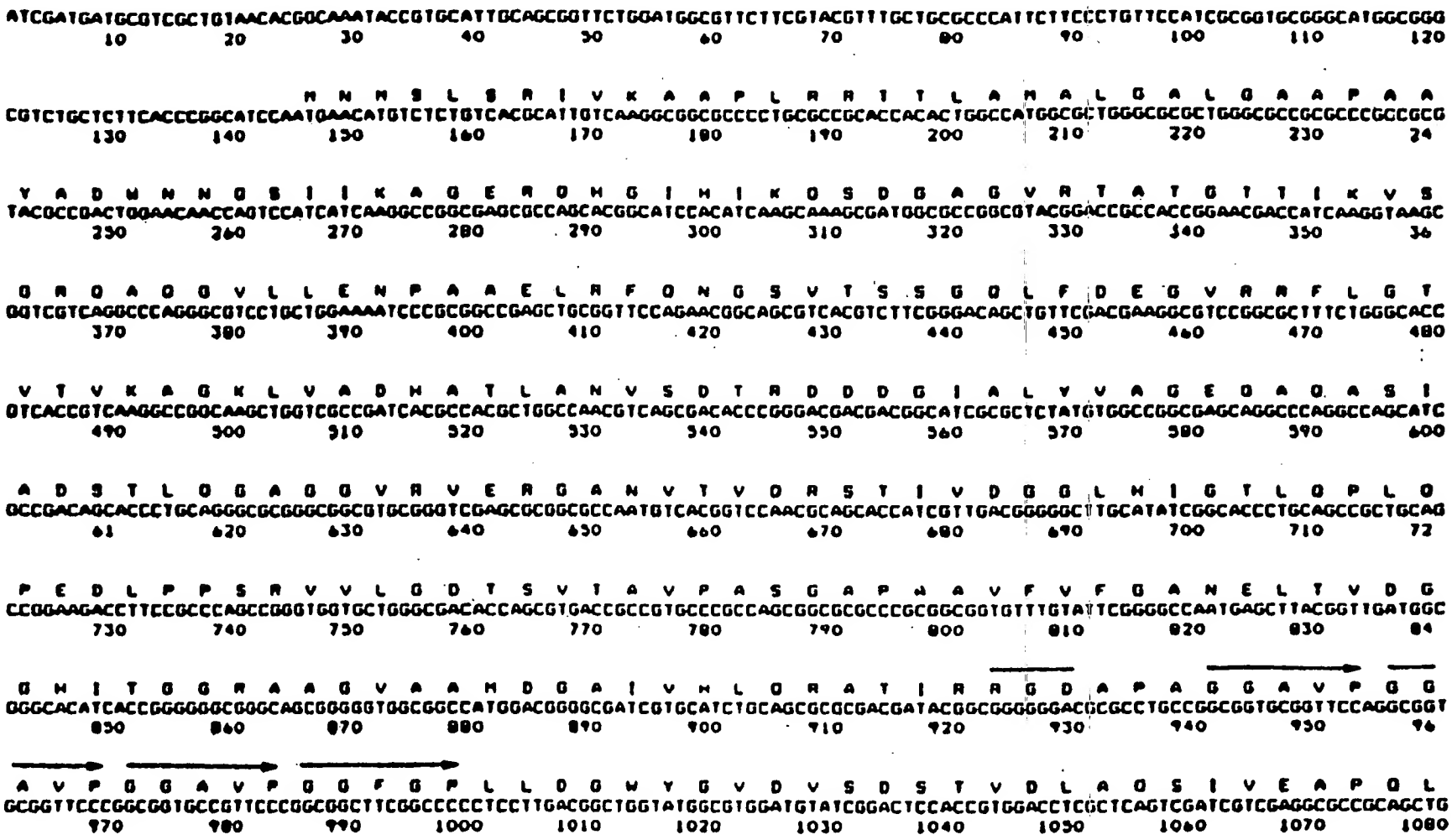
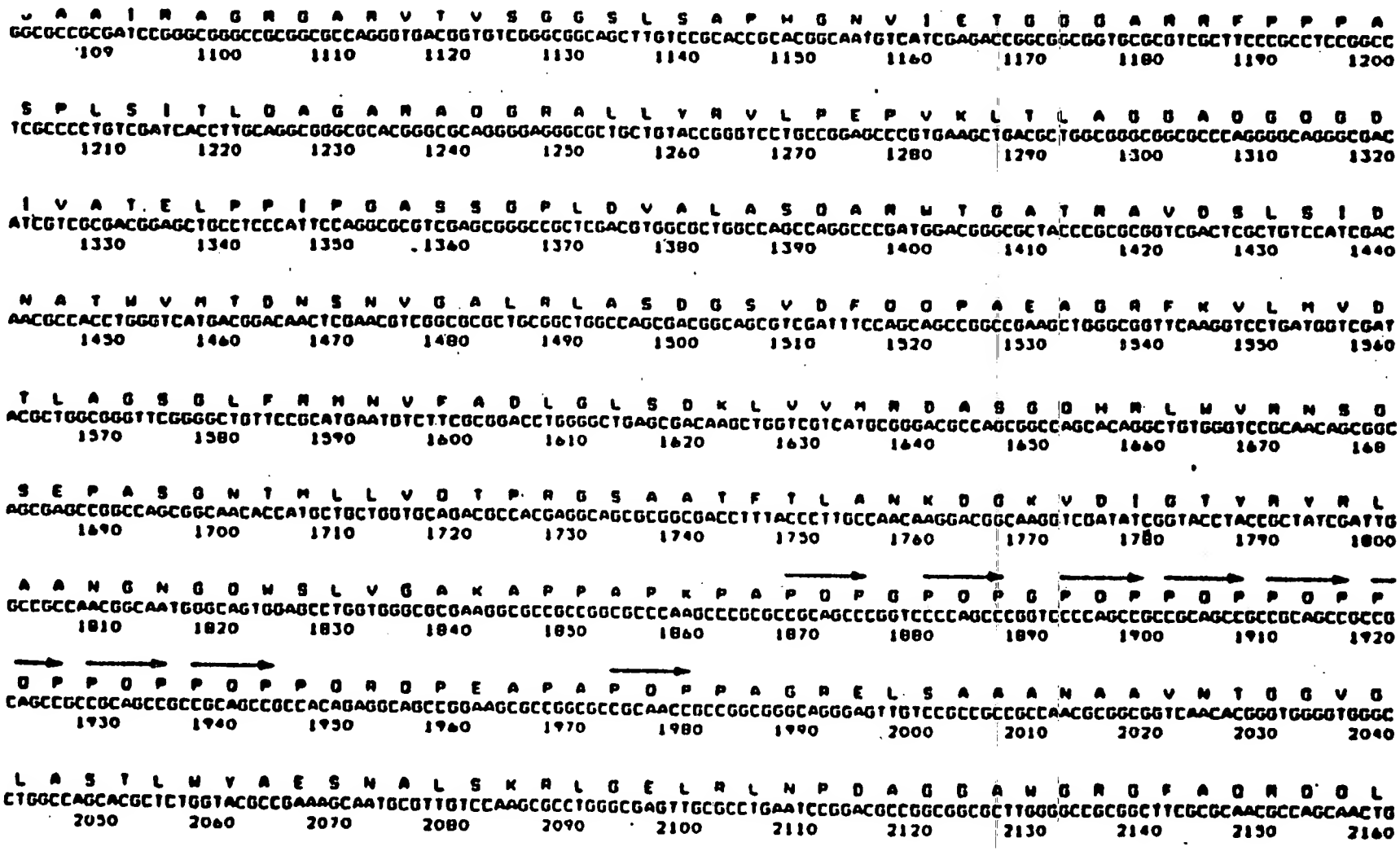
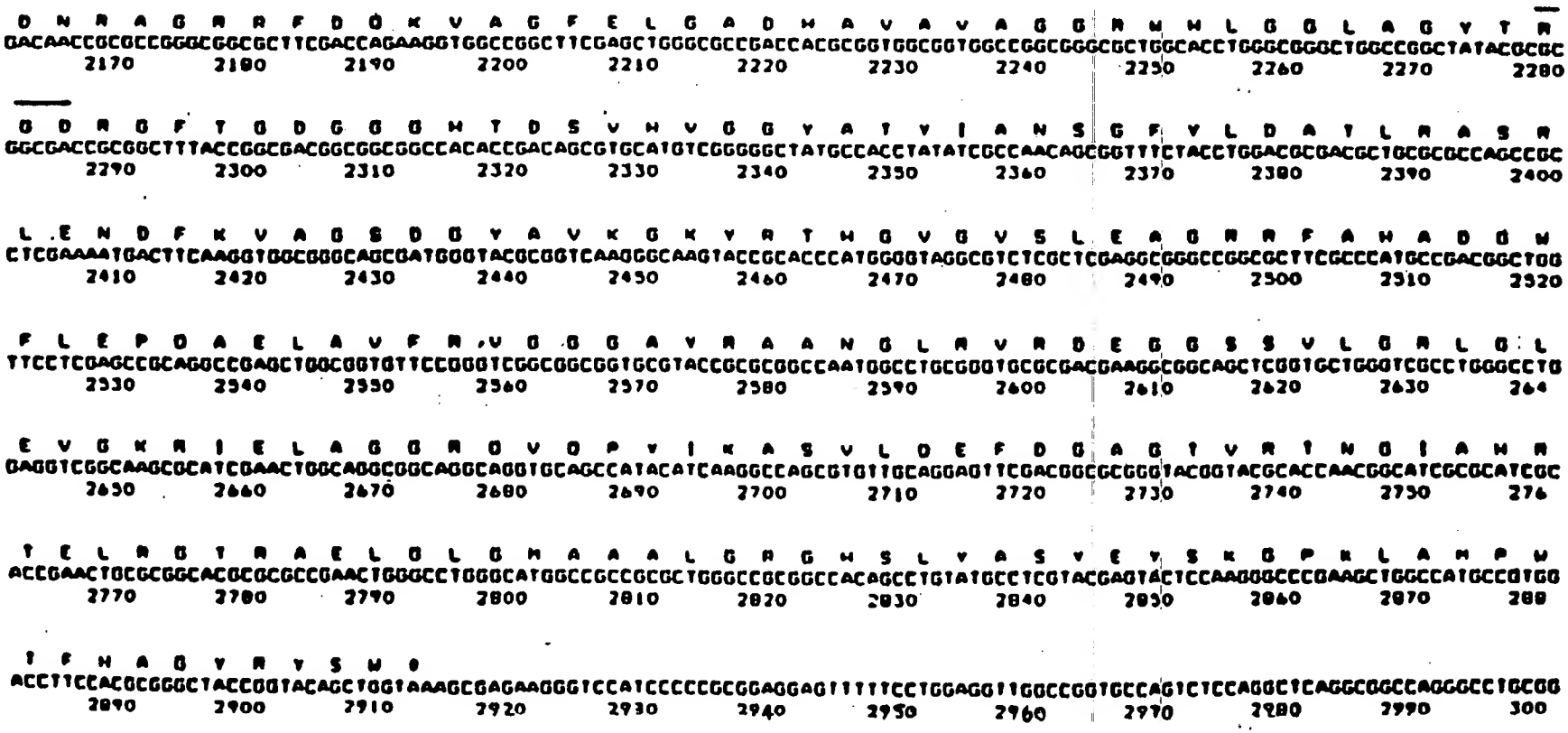


Fig.1 Cont (1).



SUBSTITUTE SHEET

Fig.1 Cont (2).



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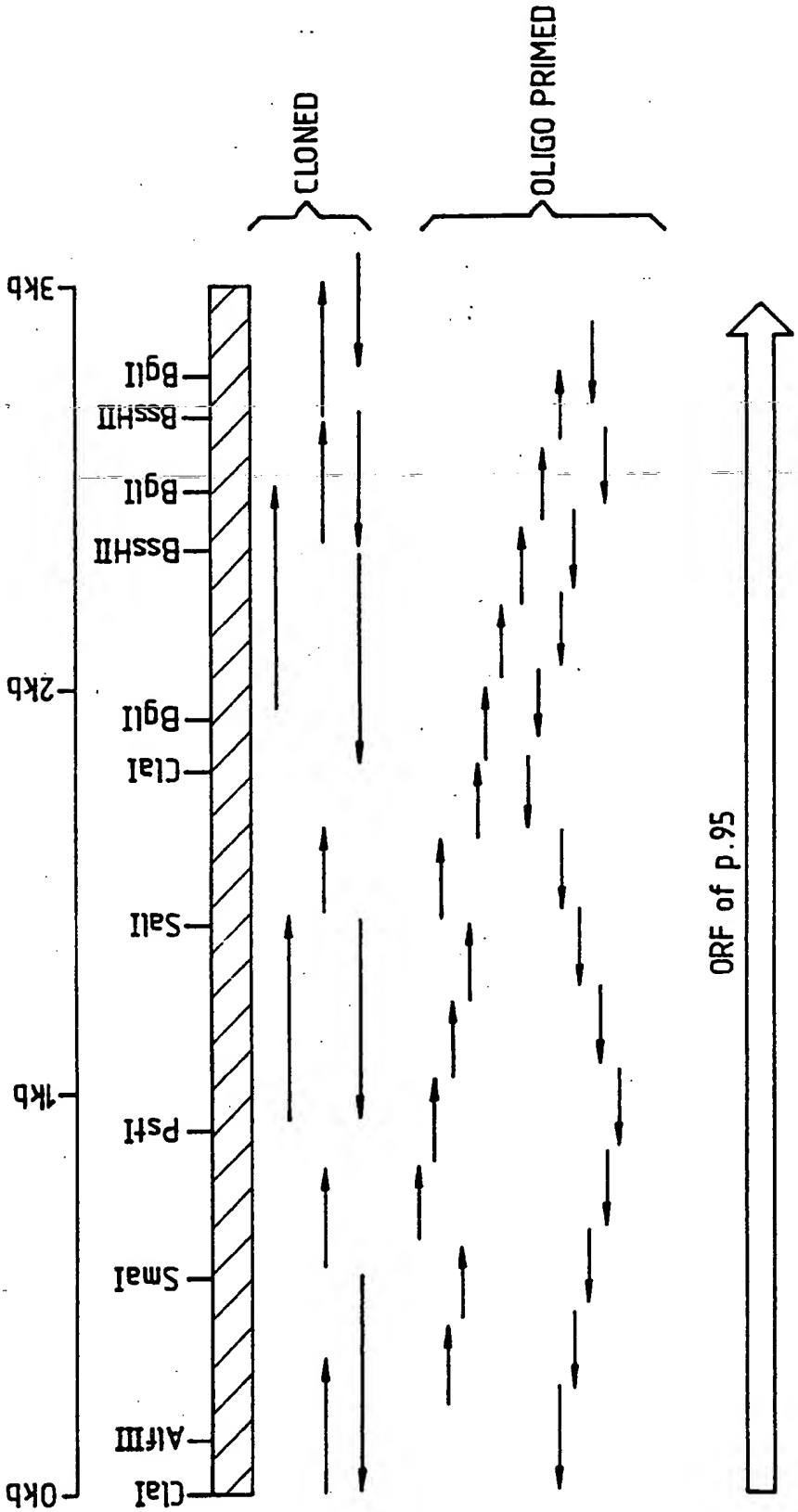
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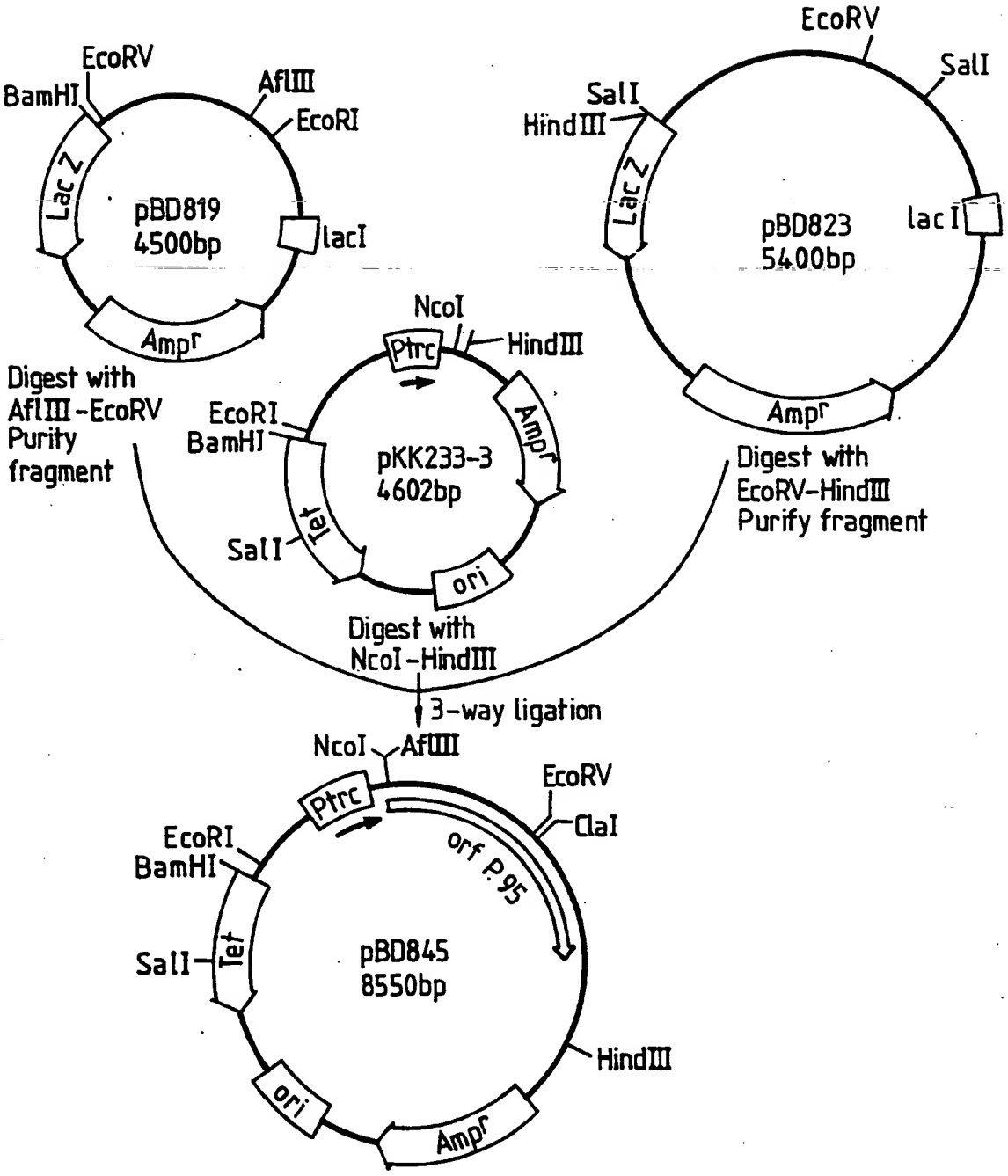
ORF of p.95

Fig. 2.

Fig.3.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EcoRV
SalI
SalI
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Digest with
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Purify fragment
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Ptrc
HindIII
EcoRI
BamHI
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ori
Ampr

INTERNATIONAL SEARCH REPORT

International Application No PCT/GB 91/02302

I. CLASSIFICATION OF SUBJECT MATTER (if several classification symbols apply, indicate all)[6]

According to International Patent Classification (IPC) or to both National Classification and IPC

IPC5: C 07 K 15/04, A 61 K 39/10, C 12 N 15/31

II. FIELDS SEARCHED

Minimum Documentation Searched[7]

Classification System	Classification Symbols
IPC5	C 07 K; A 61 K; C 12 N

Documentation Searched other than Minimum Documentation to the Extent that such Documents are Included in Fields Searched[8]

III. DOCUMENTS CONSIDERED TO BE RELEVANT[9]

Category *	Citation of Document,[11] with indication, where appropriate, of the relevant passages[12]	Relevant to Claim No.[13]
P,X	WO, A1, 9115571 (THE WELLCOME FOUNDATION LIMITED) 17 October 1991, see especially fig 1c	1-16
P,A	EP, A1, 0425082 (THE WELLCOME FOUNDATION LIMITED) 2 May 1991, see the abstract, claim 1	1-16
P,X	Dialog Information Services, file 154, Medline 85-92 Dialog accession no. 07732771, Medline no. 91251771, Li LJ et al: "P. 70 pertactin, an outer-membrane protein from Bordetella parapertussis: cloning, nucleotide sequence and surface expression in Escherichia coli", & Mol Microbiol Feb 1991, 5 (2) p409-17	1-16

* Special categories of cited documents:[10]

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IV. CERTIFICATION

Date of the Actual Completion of the International Search	Date of Mailing of this International Search Report
19th March 1992	31 MAR 1992
International Searching Authority	Signature of Authorized Officer
EUROPEAN PATENT OFFICE	MISS T. TAZELAAR

III. DOCUMENTS CONSIDERED TO BE RELEVANT (CONTINUED FROM THE SECOND SHEET)

Category *	Citation of Document, with indication, where appropriate, of the relevant passages	Relevant to Claim No
A	Biotec-Nol gy, vol. 8, November 1990, A.J. Mak ff et al.: "Protective surface antigen P69 of bordetella pertussis: its characterization and very high level expression in escherichia coli ", see page 1030 - page 1033 see p 1030 col. 2, lines 1-11 —	1-16
A	Proc. Natl. Acad. Sci., vol. 86, May 1989, I.G. Charles et al.: "Molecular cloning and characterization of protective outer membrane protein P.69 from Bordetella pertussis ", see page 3554 - page 3558 see especially p 3554, col. 1, lines 42-50 —	1-16

ANNEX TO THE INTERNATIONAL SEARCH REPORT ON INTERNATIONAL PATENT APPLICATION NO. PCT/GB 91/02302

SA 54813

This annex lists the patent family members relating to the patent documents cited in the above-mentioned international search report. The members are as contained in the European Patent Office EDP file on 28/02/92
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Patent document cited in search report	Publication date	Patent family member(s)	Publication date
WO-A1- 9115571	17/10/91	NONE	
EP-A1- 0425082	02/05/91	JP-A- 3236399	22/10/91

For more details about this annex : see Official Journal of the European patent Office, No. 12/82

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